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14. ABSTRACT
The primary objective of this research effort is the development of noninvasive imaging method to assess branched-chain amino acid metabolism (known to be modified in prostate cancer [PC]) to distinguish malignant from healthy tissue. The approach is to use MRSI of hyperpolarized ^{13}C-ketoisocaproic acid (KIC) to interrogate its conversion to leucine as catalyzed by branched-chain aminotransferase (BCAT). During this funding cycle, we:

1. Developed a high-throughput assay for assessing in vitro BCAT activity.
2. Demonstrated BCAT activity in TRAMP mouse models was significantly lower than that found in human PC.
3. Searched for an animal model more closely mimicking human metabolism by assessing four prostate cancer cell lines: PC-3, DU-145, LNCaP and LAPC-4. The PC-3 cells had the highest BCAT activity, although still appreciably lower than human PC.
4. Performed hyperpolarized in vivo MRS studies on PC-3 xenografts. Although the xenograph BCAT activity was 2.5 fold higher than cells alone (approaching human levels), the tumors grew very poorly (volumes ≤ 0.2 cc, as compared to the expected > 1 cc) and were inadequate to yield sufficient SNR for the in vivo MRS studies.
5. Initiated additional xenografts studies as well as investigation into other PC metabolic pathways.

15. SUBJECT TERMS
Hyperpolarized ^{13}C-MRS, metabolism, imaging, KIC, leucine, branched-chain amino acid metabolism, BCAT

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In Vivo Imaging of Branched-Chain Amino Acid Metabolism in Prostate Cancer

Introduction

Prostate cancer is the second-leading cause of cancer deaths among American men,[1] but in recent years, the validation of novel biomarkers has transformed the detection, prognostication and treatment of the disease.[2] Unfortunately, despite these advancements, nearly 20% of current prostate biopsies result in false negatives,[3] and there remains no reliable indicator for establishing the aggressiveness of a particular prostate tumor.[4] These deficiencies have resulted in painful biopsies, over-treatment and undesired side effects (e.g. impotence) for patients that possess tumors that will not be a health risk in their lifetime. Conversely, aggressive neoplasms may not be properly treated until they reach an advanced stage. The accurate characterization of prostate cancer via a non-invasive method would address these major clinical issues.

The primary objective of this research effort is the development a novel, non-invasive imaging technique that distinguishes malignant from healthy prostate tissue based upon their distinctive metabolic profiles. To this end, the strategy is to validate in vivo MRSI of hyperpolarized ^{13}C-ketoisocaproic acid (KIC) as a transformative method for guiding treatment and regulation of prostate cancer. This approach relies upon the ability of KIC to interrogate pathways of branched-chain amino acid (BCAA) metabolism, which are known to be modified in the tumor-bearing state. For example, recent reports have demonstrated the critical role of BCAAs in the proliferation of tumorgenic prostate tissue.[5] In particular, many of the features of BCAA metabolism in cancerous tissue are generally characterized by altered BCAA availability and elevated rates of BCAA oxidation.[6]

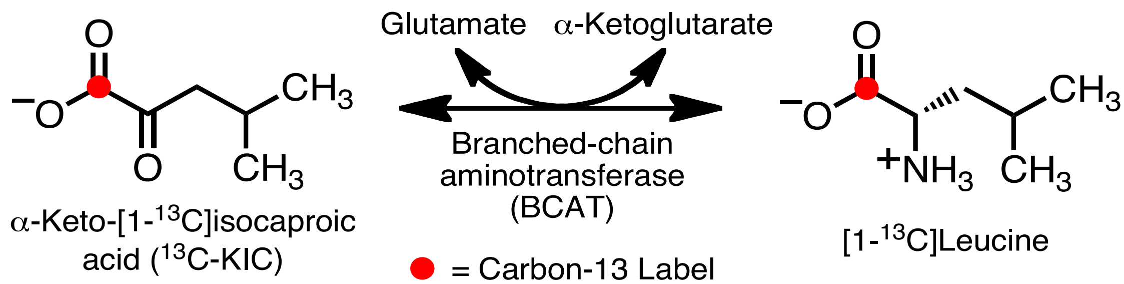

Figure 1. Metabolism of [1-^{13}C]KIC to [1-^{13}C]Leucine via the enzyme BCAT

Body

In order to determine the ideal animal model for investigating BCAA metabolism in prostate cancer, branched-chain amino acid aminotransferase (BCAT) enzymatic activity assays were performed on various tissue sources. BCAT catalyzes the initial reversible step of branched-chain amino acid catabolism[7] (Figure 1), and this enzyme has been implicated as a potential biomarker for a variety of carcinomas.[8] For measurement of BCAT activity, radiometric end-point methods have been traditionally employed,[9] but recent reports have introduced spectrophotometric methods for determining enzymatic activity.[10] Although both processes are straightforward to perform, they are, unfortunately, laborious and/or often times rely upon non-commercially available enzymes. Currently, only a limited number of protocols allow for continuous monitoring of the course of the transamination reaction, which may mitigate experimental errors from endpoint measurements.[11] However, these assays are time consuming as they require multiple experiments to yield the enzymatic activity of a single tissue sample.

In our studies, we require an accurate, high throughput method for analysis of various prostate cancer sources; therefore, we initially developed a spectrophotometic, 96-well plate assay that is amenable for determination of BCAT activity of numerous tissues in short order (Figure 2).[12] In this process, BCAT activity is assessed through coupling the transamination reaction to two enzymatic processes: (1) conversion of alanine to pyruvate via alanine aminotransferase (ALAT) and (2) the subsequent reduction of pyruvate to lactate via lactate dehydrogenase (LDH). Importantly, the last step in the sequence results in the oxidation of NADH to NAD^+ (Nicotinamide adenine dinucleotide), whose absorbance at 334 nm can be optically detected. In the assay, each well of a 96-well plate is charged with the appropriate concentration of metabolite, enzymes and tissue homogenates. Then, KIC (2.1 mM) is added to begin the experiment, and an analogous volume of water is charged into the corresponding control wells. BCAT activity is assessed through averaging the change of NAD^+ absorbance over the initial rate period while compensating for controls. Overall, this high throughput assay allows for the determination of BCAT activity from several tissue sources in a rapid and reliable fashion.

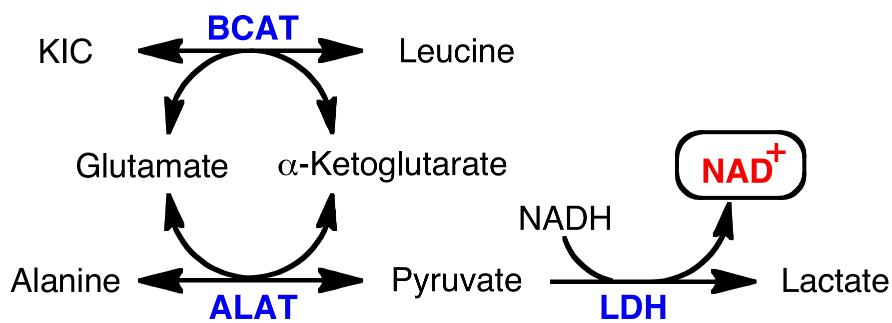

Figure 2. Spectrophotometric BCAT enzymatic activity assay

Assay Conditions
KIC: 2.1 mM
Glutamate: 300 mM
Alanine: 200 mM
NADH: 0.20 mM
PLP: 0.10 mM
ALAT: 500 uM/min
LDH: 1000 uM/min
Homogenate (BCAT): 50 - 500 g protein

The TRansgenic Adenocarcinoma of Mouse Prostate (TRAMP) model has become an indispensable tool for characterization of molecular mechanisms involved in the initiation and progression of prostate cancer.[13] A series of enzymatic assays were performed in order to determine whether the TRAMP mouse model mirrored human prostate cancer in terms of BCAT activity and, in turn, would be suitable for in vivo MRSI studies with hyperpolarized ^{13}C-KIC. In these ex vivo experiments, the TRAMP prostate tissue was found to possess a disappointingly low BCAT activity of 0.84 ± 0.17 U/gram of protein (Figure 3). For comparison, protein extracts from human prostate tumor samples were obtained. The corresponding homogenates displayed an increased overall level of BCAT activity (2.37 ± 0.64 U/gram of protein) relative to the TRAMP mouse model.

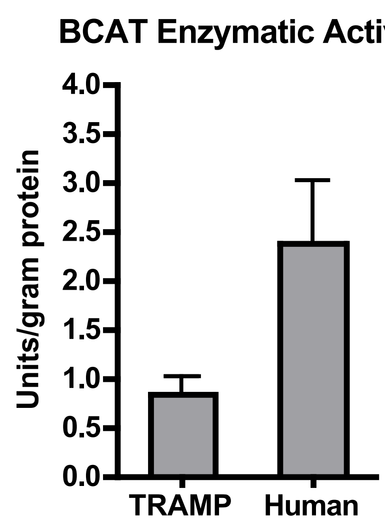

Figure 3. Comparison of BCAT activity in TRAMP mouse and human prostate cancer tissue

Although the TRAMP model has been used extensively to study prostate cancer, BCAA metabolism in this model does not appear to mimic human prostate tumors, in respect to elevated BCAT activity. In an effort to obtain an animal model that possesses higher levels of BCAT activity, a series of human prostate cancer cell lines were examined that could be utilized for the preparation of the corresponding xenograft tumor models. Four cell lines were subjected to the BCAT enzymatic assay protocol: PC-3, DU-145, LNCaP and LAPC-4.[14] In these experiments, the human prostate cancer cell line, PC-3, was identified as a potential basis for xenograft preparation as it displayed the highest level of BCAT activity (1.04 ± 0.40 U/gram of protein) (Figure 4). In addition, the DU-145 cell line was found to have comparable BCAT activity (0.84 ± 0.35 U/gram of protein) to the TRAMP mouse model. However, only low levels were detected in vitro from the LNCaP and LAPC-4 cell lines.

Xenograft models of human prostate cancer provide the opportunity to validate the applicability of in vivo MRSI of hyperpolarized ^{13}C-ketoisocaproic acid (KIC) as a powerful tool for assessing characteristics of prostate tumor metabolism. Initially, two strains of the PC-3 cell line were utilized in the preparation of xenografts: the parental cell line (PC-3) and a liver metastasized line (PC-3M). Tumors were induced on either flank of nu/nu nude mice through the subcutaneous injection of two million cells in a PBS/matrigel (50:50) medium. In general, PC-3-based xenografts showed relatively slow tumor progression in vivo and a steady growth rate of 5 mm^3/day was observed. Tumors were imaged upon reaching a size of roughly 200 mm^3 (circa 6 weeks post injection). Unfortunately, PC-3M-derived xenografts displayed instability for sustained tumor development. In two of three mice examined, tumor regression was observed after two weeks, and only one mouse provided a tumor >150 mm^3 for further analysis.

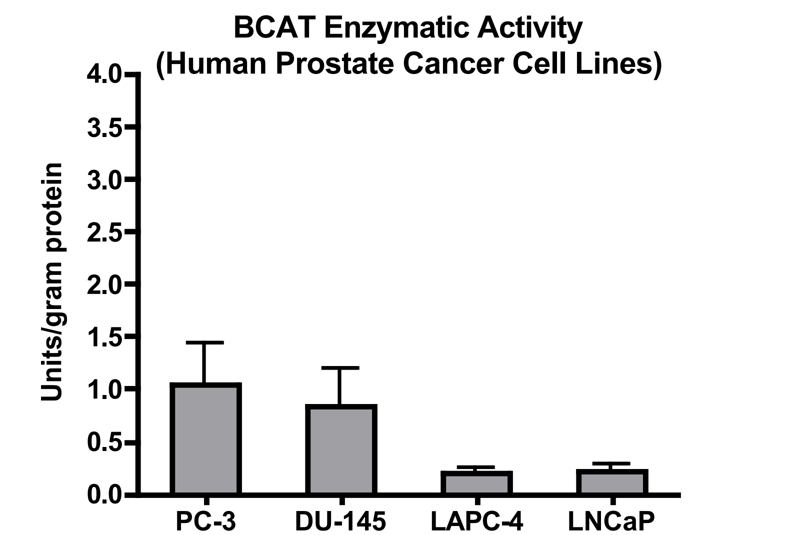

Figure 4. Comparison of BCAT activity in human prostate cancer cell lines

In vivo MRS studies were conducted on the human prostate cancer cell line (PC-3 and PC-3M) xenografts utilizing a clinical 3T GE Signa MRI scanner equipped with a high-performance gradient set insert optimized for small animal imaging. The mice were anesthetized with 1-3% isoflurane in oxygen (~1.5 l/min), a tail vein catheter was inserted into each mouse, and the animal placed in a custom-build dual-tuned ^{13}C/^{1}H quadrature birdcage RF coil (60-mm diameter) centered in the scanner bore. Body temperature was monitored using a fiber optic rectal probe and controlled using a temperature-controlled forced air heating system. In addition, breathing was monitored using a small animal respiratory monitoring hardware and software system with heart rate and O_2 saturation recorded using a pulse oximeter. Within a given scanning session, each mouse received one bolus injection of DNP hyperpolarized ^{13}C-KIC (dose of 0.3 ml, 40 mM at an injection rate of 0.025 ml/s) followed 1.5 hrs later by another injection of ^{13}C-KIC (0.3 ml, 40 mM) containing unlabelled leucine in the dissolution buffer. Conventional MRI was used to select the tissue volume of interest.

Metabolite production was analyzed in the both PC-3 and PC-3M xenografts after each injection of hyperpolarized ^{13}C-KIC. Administration of 40 mM ^{13}C-KIC showed no observable toxicity effects. However, in vivo leucine production was not detected in the prostate cancer tumors. Potentially, the low level of BCAA production could be as result of poor vascularization of the tumors.[15] Thus, a limited volume of tracer would enter the tumor, and consequently a lower level of metabolic products would be observed.

Following the MRS studies, the mice were euthanized, and the tumors were removed in order to determine BCAT enzymatic activity. These results are summarized in Figure 5. Interestingly, a greater than 2.5-fold increase in activity was detected from the PC-3-derived xenografts in respect to the in vitro results. A similar increase in xenograft BCAT activity has been previously observed.[16] Overall, PC-3-based xenografts displayed a 3.81 ± 1.27 U/gram of protein level of BCAT activity, while PC-3M xenografts also showed high enzymatic levels (2.47 ± 1.01 U/gram of protein).

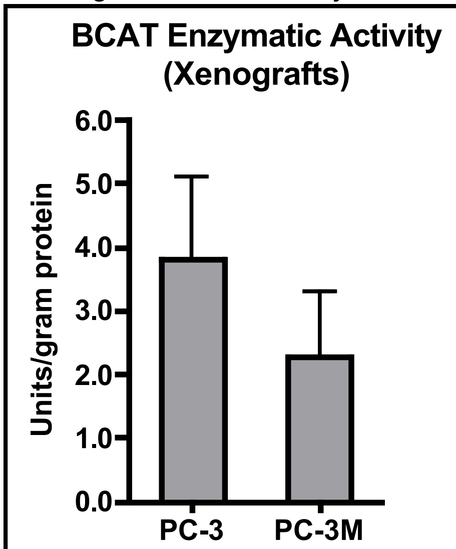

Figure 5. BCAT activity in PC-3-based xenografts

Xenografts-based upon human prostate cancer cell lines appear to be displaying BCAT activities that are comparable to anticipated human levels. In future studies, we will be generating further PC-3 xenografts for imaging studies. Several strategies for increasing the level of detectable KIC metabolism will be initiated. First, tumor sizes will be increased prior to imaging studies. Larger tumors require a significantly greater vascularization and association with the organism for supply of nutrients required for sustainable growth. Ideally, this increase in vasculature will allow for a greater volume of the hyperpolarized ^{13}C-KIC to enter the tumor and, correspondingly, be metabolized to the downstream products. Lastly, a second xenograft model based upon DU-145, a cell line shown to have relatively high level of BCAT activity, will also be pursued.

Key Research Accomplishments

1. Developed a high-throughput assay for assessing in vitro BCAT activity.
2. Demonstrated BCAT activity in TRAMP mouse models was significantly lower than that found in human prostate cancer (PC).
3. Searched for an animal model more closely mimicking human metabolism by assessing four prostate cancer cell lines: PC-3, DU-145, LNCaP and LAPC-4. The PC-3 cells had the highest BCAT activity, although still appreciably lower than human PC.
4. Performed hyperpolarized in vivo MRS studies on PC-3 xenografts. Although the xenograph BCAT activity was 2.5 fold higher than cells alone (approaching human levels), the tumors grew very poorly (volumes ≤ 0.2 cc, as compared to the expected > 1 cc) and were inadequate to yield sufficient SNR for the in vivo MRS studies.
5. Initiated additional xenografts studies as well as investigation into other PC metabolic pathways.

Reportable Outcomes

Billingsley K, Josan S, Park J, Mayer D, Brooks J, and Spielman D, "Branched-chain aminotransferase (BCAT) activity in human prostate cancer and prostate cancer animal models", in preparation.

Conclusion

Our most significant finding to date is that the widely used murine prostate cancer TRAMP model does not appear to accurately reflect some of the basic metabolic processing occurring in the human disease. This is surprising given the success in using the TRAMP to examine tumor energy metabolism, which has recently be shown to be similar in the first prostate cancer human trial with hyperpolarized ^{13}C-pyruvate[17]. We've also extending findings showing very different enzyme activity levels in xenograph as compared to that seen in the corresponding cell lines. This highlights the importance of view cancer in general, and prostate cancer specifically, as metabolic disorder involving complex interactions among tumor cells, rather than a disease cause by a small set of genetic abnormalities. Future work will involve extending our search for the best animal model that most accurately mimics the metabolic alternations found in human prostate cancer, focusing on metabolic pathways assessable by in vivo imaging using MRS of hyperpolarized ^{13}C-labeled substrates. This includes a primary focus on nitrogen balance via branched-chain amino acid metabolism. In addition, we would like to expand our investigations to other novel hyperpolarized substrates yielding unique insights into in vivo prostate cancer metabolism, however such an extension of scientific aims will only be conducted subsequent to approval by our Army Contracting Officer Representative, Dr. Melissa Cunningham.

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Appendices

none